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NO. 413

STUDIES ON THE MECHANISM OF ACTION OF THE ENHANCED PROTECTIVE EFFECT OF MULTIPLE VS A SINGLE DOSE OF HI-6 AGAINST SOMAN POISONING (U)

by

Paul M. Lundy and Brian Hand

Project No. 13D20

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ABSTRACT

Atropine pretreated rats given soman (650 μg/kg) survived an average 3.2 min. HI-6 (125 mg/kg, i.p.) increased survival time of all animals usually 3 – 6 h while multiple doses of HI-6 given post soman completely protected rats for at least 14 days. Soman treated animals given one dose of HI-6 developed short-lasting convulsions, followed by a convulsion free period then in about 1 1/2 h by a gradual return of severe tremors. A second dose of HI-6, unlike diazepam, quickly abolished the tremors.

ABSTRACT (Cont'd)

Diaphragm AChE activity was higher than control values following 1 dose of HI-6 1/2 h post soman but not at subsequent time intervals, while diaphragms from animals treated with HI-6 several times were higher than controls for at least 3 h. AChE activity remained virtually unchanged in all brain areas examined following either regimen. Skeletal muscles removed from animals which had developed delayed tremors had virtually no AChE activity nor did muscles from animals given a second dose of HI-6 which stopped the tremors. The results indicate that multiple HI-6 doses afford much better protection from soman than one dose. This enhanced protection may be due partially to the maintenance of AChE activity in the diaphragm for long periods and additionally to a direct effect on skeletal muscle resulting in improved respiratory function.

These results indicate that HI-6 may be even more useful against soman poisoning than previously recognized if the dose schedule is altered. When future studies are planned to test the effects or effectiveness of HI-6 in other species or in human trials, the factor of multiple injections over long periods of time should be a major consideration.

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INTRODUCTION

The beneficial effect of HI-6 on organophosphate toxicity, particularly soman toxicity, is now well established (Oldiges and Schoene, 1970; Wolthuis and Kepner, 1978; Lipp and Dola, 1980). Although HI-6 has an AChE reactivating oxime function, its mechanism of action against soman is unclear. For example, various studies have indicated that soman induced mortality is due to central respiratory paralysis (De Candole *et al.*, 1953; Meeter and Wolthuis, 1968; Adams, Yamamura and O'Leary, 1976) however HI-6 appears to be unable to enter the brain in concentrations sufficient to reactivate brain AChE inhibited by soman (Clement, 1981; Sterri, Lyngaas and

Fonnum, 1983; Lundy and Shih, 1983) nor does it inhibit the rise in brain ACh levels from soman treated animals (Lundy and Shih, 1983). HI-6 on the other hand *in vivo* does reactivate brain AChE inhibited by other organophosphates as well as soman inhibited peripheral AChE (Clement, 1982; Harris *et al.*, 1981; Sterri *et al.*, Lundy and Shih, 1983) as well as reactivating AChE *in vitro* (Schoene, Steinhanses and Oldiges, 1983). Injections of HI-6 directly into cerebral ventricles failed to reactivate soman inhibited brain AChE perhaps because its own toxicity by this route precluded the use of concentrations sufficient to observe the predicted effect (Lundy and Shih, 1983).

Since the half life of HI-6 in the periphery of experimental animals is short (Ligtenstein and Kossen, 1983) attempts were made to maintain "therapeutic" levels of HI-6 and to observe the effects of such treatment on the protective ratio $\frac{(LD_{50}\text{ soman + treatment})}{(LD_{50}\text{ soman alone})}$ of HI-6 by giving the compound in multiple doses. In addition to the dramatic increase in the protective ratio which was observed, other previously unreported effects of the oxime were noticed and studied.

This report examines the central and peripheral AChE activity in relation to the enhancement of survival of soman treated animals treated once or several times with HI-6. In addition these studies have shown a dramatic antitremorigenic activity of HI-6 following a second injection. Further aspects of the mechanism of action of HI-6 against soman poisoning are discussed.

METHODS

Animal Preparations

Male, Sprague-Dawley rats, weighing 230 – 260 g were used in all experiments. The animals were housed in temperature controlled quarters with fixed light dark cycles. Laboratory rat chow and water were freely available. All experiments, i.e. initial injections, were begun between 0900 and 1100 h each day.

Tissue Removal and LD_{50} Determinations

Initial LD_{50} studies were carried out over various time intervals up to 2 weeks with soman alone, s.c., or soman plus atropine SO_4 (i.p., 30 min prior to soman). The initial dose of HI-6 (all cases 125 mg/kg, i.p.), was given 30 – 60 s prior to soman. For biochemical determinations animals were sacrificed 1/2 h, 1 1/2 h, 3 h or 5 h post

soman. The group given multiple injections received further doses of HI-6 at 1/2 h (sacrificed and AChE determinations done at 1 1/2 h), at 1 1/2 h (sacrificed at 3 h), at 3 h (sacrificed at 5 h). For the 2 week LD_{50} studies they were injected again at 6 h post soman. Tissues from control animals (soman or soman plus atropine sulphate) were removed at death. Mortality was recorded each hour for 6 h and then every 24 h where applicable. Brain tissue was divided into four areas: cortex, hippocampus, striatum and brain stem for AChE analysis. In addition, in some studies both the rectus abdominis and the tensor fasciae latae were removed and their AChE activity measured.

AChE Assay

Assays were carried out according to the radiometric method of Siakotos, Filbert and Hester (1969) utilizing [C^{14}] ACh iodide (1.7 mCi/mmol; New England Nuclear) as substrate. Briefly the peripheral muscles were removed and homogenized in 10% w/v homogenization buffer with a polytron homogenizer for 15 s. Brain tissue was homogenized in 1% w/v homogenization buffer in a teflon homogenizer. All samples were incubated for 5 min and an aliquot of the incubation mixture was added to 10 mL of scintillation fluid (0.5 g Bis MSB; 5 g PPO; 330 mL Triton X-100 q.s. 1 L with toluene) and counted in a Beckman LS 9800 scintillation spectrometer.

Tremor Measurement

Tremors were measured in a "Vibromex" (Model 82-1) (Columbus Instruments). Animals which were tremoring severly were placed in the clear cage sitting on the platform over a sensitive pressure transducer which monitors the frequency components of animal movements. Animals monitored in this study were virtually free of any activity except for various degrees of intermittent tremor activity. The meter was adjusted by setting a ratio of two frequencies, one consisting of a low frequency component which are normal components of walking, etc. and a higher frequency which consists of tremorigenic activity, to a point just inside the higher frequency activity.

The "Vibromex" then measures and prints out the number of individual tremors which consist of the predominant higher frequency ratio, of more than 0.1 s duration. It also measures the time in each minute during which the animals activity is above the preset ratio and also collects the number of individual tremors or total tremorigenic activity/time period.

Animals given soman ($6 \times LD_{50}$ or 650 μg/kg) and HI-6 invariably begin to develop tremors after about one hour which become increasingly severe, becoming maximal at about 2 h. At this point in time animals were placed in the tremor monitor and a 5 min control reading was taken followed by a second injection of HI-6 and a further monitoring period. In some experiments animals were given diazepam (2.5 mg/kg or 5 mg/kg), monitored for ten minutes and subsequently given the second dose of HI-6 and monitored for a further time period.

Drugs and Chemicals

Atropine sulphate purchased from Sigma Chemical Company (St. Louis, MO), soman and HI-6 [[[(4-aminocarbonyl)pyridino]methyl]-2-[(hydroxyimino)-methyl]-pyridinium dichloride, were synthesized by the Chemistry Section, Defence Research Establishment Suffield. Soman was supplied each month and was periodically examined to ensure purity. All drugs were dissolved in 0.9% saline. Diazepam was a generous gift of Hoffman LaRoche and it was dissolved for injection in PEG.300.

Antitremorigenic Effect of HI-6

Animals were given the usual atropine pretreatment and HI-6 30 – 60 s prior to a challenge with $6 \times LD_{50}$ soman. After two hours, one group of animals with severe tremors were sacrificed and parts of two peripheral skeletal muscles (tensor fasciae latae and rectus abdominis) which demonstrated obvious abnormal activity, were removed for AChE determination. Another group of tremoring animals was given a second dose of HI-6 and 5 min later when tremor/fasciculations had ceased the skeletal muscles were removed and assayed for AChE activity. Whole brain AChE activity in these two groups was also compared.

Statistics

LD_{50} values were calculated by the probit procedure of Finney (1971).

The significance of the difference of AChE values at the various time intervals were calculated using the Student-Newman-Keuls procedure to measure the significance of the differences among various means. Differences between control and treated, between the various time intervals and the two procedures, single treatment vs multiple treatment at two levels of significance were tested. Significance was determined for the purposes of the results presented here at $p < 0.05$ between the various groups.

RESULTS

Animals were given various treatments and their survival rate followed closely for 6 h, then at 24 h periods up to 14 days. The results of these studies are depicted in Figure 1. All animals received atropine sulphate 17 mg/kg 1/2 h prior to soman. Rats given 650 μg/kg (6 × LD_{50}) of soman all died within 5 min (■) (avg. 3.2 min). Animals which received one injection of HI-6 were given soman 650 μg/kg (○) or 900 μg/kg (●) and the mortality plotted hour by hour (10 – 12 animals/group). It is obvious that the HI-6 treated animals survived much longer periods of time than did animals which received no HI-6 and that one dose of HI-6 was extremely beneficial for periods at least up to 3 h. In a number of similar experiments (results not shown) the survival time of the animals was usually somewhat longer than depicted here, almost all animals surviving at least 3 h. Two other groups of animals were given the same two doses of soman but were given additional doses of HI-6 post soman. Following 650 μg/kg soman, one group received four additional doses of HI-6, 1/2 h, 1 1/2 h, 3 h and 6 h post soman. The percent mortality vs time was plotted for 14 days (△). These animals all survived 14 days. All eight animals given 900 μg/kg soman and multiple HI-6 (□) survived 6 h. Within 24 h 7/8 animals died and the remaining survivor lived 24 – 48 h. A third group of animals given 900 μg/kg soman received HI-6 at 1/2, 1, 2, 3, 4, 5 and 6 h and 58% of these animals survived the two week observation (▲) period and appeared healthy, gaining weight, etc. during this time.

The animals in either HI-6 treated group responded to the large doses of soman by convulsing for a short period of time and then becoming for the most part, quiescent. In animals receiving one dose of HI-6 tremors began to return after a period of about 1 h. These animals became increasingly intoxicated as judged by external signs, and eventually a proportion died within 6 h. No return of severe toxic signs were evident up to 6 h in the animals which had received 5 doses of HI-6. Following 14 days, animals which survived appeared normal although brain AChE activity was about 50 percent of normal activity while diaphragm AChE activity had returned to normal control values (unpublished observations).

AChE activity in various brain areas was measured at death in atropine pretreated rats given soman 650 μg/kg (control) (Table 1). The average time taken until death was 3.2 min. The AChE activity of four brain areas was significantly and quite uniformly depressed in all areas examined (Table 1). In animals which had received one dose of

HI-6 (125 mg/kg) the AChE activity was not found to be significantly different from control values in any of the brain areas at any time post soman (1/2 h, 1 h, 3 h, 5 h).

The AChE activity measured in brain areas of animals which received multiple doses of HI-6 was also not higher than either animals receiving no HI-6 or one dose of HI-6. Only the AChE activity of hippocampal homogenates was increased significantly, 3 h post soman. This change was considered to be of dubious physiological significance because of its transient nature and the relatively small size of the change.

Table 2 shows the AChE activity in diaphragm muscle measured in the same rats from which brain AChE activity had been measured (see Table 1). One dose of HI-6 raised the AChE activity significantly above the activity found in soman (control) animals at 1/2 h. However after the initial increase in AChE activity at the first time period (1/2 h) the activity fell to control values at the subsequent time periods (1 1/2, 3 and 5 h). Some contrasts were found between the AChE activity in diaphragms from animals which received one dose of HI-6 and the activity found in diaphragms of animals given multiple injections. In animals given 4 doses of HI-6, AChE activity was significantly greater than in control animals but in contrast to once treated animals it remained significantly elevated at 1 1/2 and 3 h. By 5 h, the diaphragm AChE activity although somewhat higher than that found in once treated animals was not significantly higher than the activity found in diaphragms from animals receiving no HI-6. The physical condition of once treated animals appeared extremely good for periods of up to or slightly beyond one hour. They then slowly deteriorated and began to show tremors at about 1 1/2 h. The tremors slowly become more severe, respiratory movements appeared to deteriorate and death followed. On the other hand the multiple HI-6 treated animals appeared in much better condition, attempted to eat and walk and appeared to survive the 6 h without further severe difficulty and certainly without further tremors.

The reappearance of tremors 2 h post soman in animals given one dose of HI-6 were also examined. One group of animals received one dose of HI-6 30 – 60 s prior to soman (○) while the second group (●) received additional HI-6 at 1/2 h and at 1 1/2 h post soman. Tremor activity was measured 2 h post soman. Results are illustrated in Figure 2. At the peak of the tremor animals manifested severe activity (about 35/min, Fig. 2A) for 15 – 20 s of each minute (Fig. 2C) while tremors (cycles) reached a five minute average of about 240 (Fig. 2B). In the group of animals which received three HI-6 doses the relative lack of tremor activity (●) is also shown clearly.

The dramatic difference in tremor activity between rats given one HI-6 dose vs a second dose is evident from the results in Figure 2D to 2F. Two hours post soman tremorigenic activity was measured in animals given one dose of HI-6. After recording their activity for 5 min they were removed from the tremor meter and given a second dose of HI-6 which quickly lowered all three parameters of activity measured by the tremor monitor. In order to determine whether this reduction in activity was centrally mediated, a potent inhibitor of soman induced seizure activity was injected in rats which were tremoring severely. Figure 3 depicts the effects of diazepam 2.5 mg/kg which caused no apparent attenuation of the tremor activity. Following a 10 min observation period, a second dose of HI-6 was administered to these animals which, as was the case in Figure 2D – 2F, resulted in marked reductions in the various tremor patterns measured from about one third to one fifth of that measured following diazepam.

The results of the previous experiments indicated that the effects of HI-6 on tremors were peripherally mediated and were apparently due to an action of HI-6 on fasciculating muscle groups. Two hours after 650 μg/kg soman in HI-6 once treated animals, two skeletal muscle groups (rectus abdominis and tensor fasciae latae, T.F.L.) were removed during the period of severe tremors and the AChE activity measured. AChE activity was measured in a second group of muscles from animals 5 min after a subsequent HI-6 injection when all obvious activity was absent. At the same time whole brain AChE activity was also measured and the results of these studies are presented in Table 3. AChE activity was not different in the two muscle groups whether they were fasciculating or 5 min after the second injection of HI-6 when they were not. There was also no change in whole brain AChE activity between animals which were tremoring and animals which were not.

DISCUSSION

HI-6 has undergone extensive study in recent years and is of particular interest because, unlike clinically available oximes such as PAM-Cl, P_2S, toxogonin or TMB4, it is an extremely useful agent when used to protect animals from soman poisoning when used in conjunction with a centrally acting cholinolytic (Shih *et al.*, 1984). It is effective in a variety of species including mice (Oldiges and Schoene, 1970), rats (Wolthuis and Kepner, 1978), rabbits (Harris *et al.*, 1981), dogs (Weger and Szinicz, 1981) and monkeys (Lipp and Dola, 1980). For reasons which are not clear, the guinea pig appears

to respond less well to the therapeutic effec s of HI-6 in comparison with the traditional oximes (Inns and Leadbeater, 1983).

Animals pretreated with atropine and given HI-6 just prior to soman (650 μg/kg) convulsed severely for a short time then became quiescent for 1 to 1 1/2 h. After this time period had elapsed tremors began to return, the severity increased and breathing became more difficult until death followed sometime between 3 and 6 h but always within 24 h post soman. Since HI-6 has a short half life in rats (Ligtenstein and Kossen, 1983), the amount of HI-6 in blood and tissues after 3 h when deaths began to occur could be expected to be relatively insignificant. By arbitrarily selecting times to re-inject the animals with additional HI-6, in order to keep the HI-6 levels relatively high, we examined the effects of two multiple dose regimens on the toxicity of two high dose levels of soman. Following 650 μg/kg soman, re-injection with HI-6 post soman at 1/2, 1 1/2, 3 and 6 h resulted in 100 percent animal survival up to 6 h and ultimately 14 days, whereas survival was much shorter when the soman dose was increased to 900 μg/kg (12 percent at 24 h). Either group of animals given multiple doses appeared to be in much better condition in the early stages after poisoning and were capable of some ambulatory activity and attempted to eat in contrast to the once treated animals which showed tremors and laboured breathing as time went on. In animals which received one initial HI-6 dose plus additional injections at 1, 2, 3, 4, 5 and 6 h the survival rate rose to about 60 percent at both 1 and 14 days. Although no direct comparisons between our previous results with HI-6 and soman can be made due to slight differences in methodology (Lundy and Shih, 1983) it was apparent that the multiple dose regimen produced much higher protection factors than that produced in the once treated animals. Thus with this dose regimen of HI-6 it is obvious that protection could be afforded to well over 9 times the LD_{50} of soman for 6 h and the protection ratio could be increased to about 9 times the LD_{50} for from one or fourteen days although, in these studies no attempt was made to determine the absolute protective ratio accurately nor to determine the maximal protective effect of HI-6. It also appears evident that the more often HI-6 was administered the better was the protection obtained. However it should be noted that this may not be the optimal regimen of HI-6 use for animal protection studies, nor do we know at the present time what protection factors would have been attained if repetitive HI-6 injections would have been made for perhaps a 24 hour period.

Two observations suggested that HI-6 may have had central effects at high doses of soman which we had not observed previously at smaller doses. A second HI-6 dose

given to animals 2 h post soman, during severe tremors immediately abolished the tremors (1 – 2 min). Secondly, rats given multiple doses of HI-6 failed to develop tremors or convulsions except in the first few minutes following soman. Initially it appeared likely to us that the very large doses of soman (650 μg/kg) may have produced effects on the blood brain barrier which would allow easier entrance of HI-6 to brain tissue. Alternately, since the half life of HI-6 in the brain has been shown to be much longer (hours) than it is in the periphery (minutes) (Ligtenstein and Kossen, 1983), consideration was given to the possibility that HI-6 could enter the brain in small quantities (Ligtenstein and Kossen, 1983) following each injection, thus becoming cumulative and ultimately reaching AChE reactivating concentrations at some point in time. However no evidence of either such effect was obtained from direct AChE measurements from a number of brain areas. Following 650 μg/kg soman, a single dose of HI-6 failed to reactivate central AChE at any time period examined up to 5 h. This result is similar to those obtained at smaller doses (1 – 2 × LD_{50}) of soman (Clement, 1981; Sterri *et al.*, 1983; Lundy and Shih, 1983). In addition, multiple doses of HI-6 also failed to reactivate AChE in any brain area at any time except in the hippocampus at 3 h, this apparent reactivation being statistically significant but of doubtful physiological effect especially since the change was not evident at the next time period of measurement. These results do not on the other hand rule out the possibility that either high doses of soman might afford easier access of HI-6 to brain nor do they rule out the possibility that multiple HI-6 doses result in central accumulation subsequently producing some as yet undefined effect. They do appear to rule out a role for HI-6 in central AChE reactivation under these conditions.

Blood, lung, tissue or diaphragms from HI-6 treated animals following relatively low doses of soman (1 – 2 × LD_{50}) have been reported to have higher AChE activity than tissues from animals which received no HI-6, at time periods up to 1 h (Harris *et al.*, 1981; Clement, 1981; Sterri *et al.*, 1983). Therefore, the increased AChE activity following HI-6 combined with high doses of soman (650 μg/kg) reported here appears to be similar to previously reported results. However at the high doses of soman some interesting differences were recorded in contrast to those obtained at lower doses. The increase in AChE activity 1/2 h post soman following a single HI-6 dose was no longer statistically significant at 1 1/2 or 3 h indicating an apparent re-inhibition of AChE. This re-inhibition correlated well with the return of toxic effects although almost all of the animals lived for over 3 h, however the diaphragm AChE activity was not

significantly above control values (essentially zero). Multiple HI-6 treatment on the other hand protected or reactivated enzyme at 1/2, 1 1/2, 3 but not 5 h post soman which suggested only a partial correlation between diaphragm ACh activity and the much superior condition of the animals as well as their vastly superior survival rate. The results of other experiments in which animals were given 220 μg/kg (2 $\times$ LD_{50}) soman and no atropine showed that although diaphragm AChE activity could be maintained at elevated values, the animals although surviving longer than soman controls, still died with relatively high diaphragm AChE activity (results not shown). Three suggestions concerning the mechanism of this increased diaphragm AChE activity seem possible. The first and most unlikely is that HI-6 can reactivate at least some aged soman inhibited enzyme. The second possibility concerns a direct reaction of oxime with soman forming a phosphonylated oxime and the third alternative deals with the possible interference of the egress of soman from a previously proposed soman depot (Wolthuis *et al.*, 1981; Van Helden and Wolthuis, 1983). In any case, the protective effect appeared only partially correlated with the presence of functional AChE in peripheral muscle. This is not to suggest a peripheral mechanism of soman toxicity, on the contrary, for example we have shown that HI-6 used in conjunction with a peripheral cholinolytic (atropine methyl nitrate) was of negligible value (Shih *et al.*, 1984) in soman poisoning although survival time was somewhat prolonged. The elevated peripheral AChE activity in animals which nevertheless did not live, supports this contention. It is our belief that the protective effect of HI-6 on, for example, respiratory muscle and lung AChE (Sterri *et al.*, 1983) may result in the preservation of some reflex activity peripherally which helps maintain respiratory drive centrally.

Other results presented here strongly implicate a direct effect of HI-6 on skeletal muscle in the etiology of the increased protective effects which have been observed. Animals treated once with HI-6 had recurrant severe tremors after 2 h which were significantly reduced by HI-6 but largely unaffected by diazepam, a compound known to have marked anticonvulsant effects against soman (Lipp, 1972; Lundy and Magor, 1978; Lundy and Shaw, 1983). The results clearly indicated that much of what has been described as tremor/convulsive activity following soman, was in fact peripherally mediated muscle fasciculations. Fasciculating muscles, prior to the second HI-6 dose, had the same AChE activity as nonfasciculating muscles following a second dose of HI-6. In addition no difference in brain AChE activity was seen between these two groups of animals. The effect of HI-6 on the tremors appeared to be much too rapid to

be the result of enzyme reactivation even if reactivation could be possible 2 h after soman injection. Previous studies *in vitro* have shown undefined "direct" effects of certain oximes including HI-6 on diaphragm and intercostal muscle (Wolthuis *et al.*, 1981). There appears however, to be little correlation between the ability of these oximes to restore neuromuscular function *in vitro* with *in vivo* protective effects (French *et al.*, 1983) since oximes which have no protective effect are quite efficient at restoring soman blocked neuromuscular transmission *in vitro* by a direct effect.

We suggest that the direct effect of HI-6 on skeletal muscle *in vivo* shown here is one important component of the mechanism of its action against soman. The ability of HI-6 to block fasciculations of skeletal muscles including respiratory muscle such as the diaphragm, would aid greatly in sustaining coordinated muscle activity required to produce a "normal" respiratory pattern. In addition it is unlikely that the antitremorigenic effects of HI-6 could be due to any mechanism concerning AChE, either as mentioned earlier or as a result of inhibition of soman release from a postulated depot because of the speed of action of HI-6. In addition, there was no effect of the second HI-6 dose on muscle AChE activity. The exact effect of HI-6 on fasciculating skeletal muscle is also not yet clear, although it may be related in some manner to its inhibition of neuromuscular (Clement, 1979) or ganglionic transmission (Lundy and Tremblay, 1979) since it may have effects on nicotinic receptors.

We propose that HI-6, in multiple doses, may produce protection via initial reactivation of AChE peripherally with an additional direct action on skeletal muscle to prevent fasciculations. The results do not rule out additional effects such as an inhibition of soman egress from a proposed binding site or to a direct interaction with soman. In this study, as in previous ones, no demonstrable central cholinergic effects of HI-6 have yet been uncovered which might explain its efficacy (Lundy and Shih, 1983).

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TABLE 1

EFFECT OF SINGLE VS MULTIPLE DOSES OF HI-6 ON AChE ACTIVITY IN DIFFERENT AREAS OF RAT BRAIN

	TOTAL HI-6 DOSES	TIME OF HI-6 ADMINISTRATION	TIME TO DEATH/ SACRIFICE	AChE ACTIVITY[a]			
				BRAIN STEM	CORTEX	HIPPOCAMPUS	STRIATUM
Control			—	8.87 ± 0.13	5.18 ± 0.17	8.78 ± 0.17	34.78 ± 1.75
Soman[b]			3.2 min	1.07 ± 0.09	1.23 ± 0.05	0.91 ± 0.045	1.06 ± 0.08
Soman + Single Dose HI-6	1[c]	30 – 60 s prior	1/2 h	1.24 ± 0.04	0.96 ± 0.10	1.12 ± 0.058	1.12 ± 0.12
	1		1 1/2 h	1.33 ± 0.06	1.30 ± 0.14	1.28 ± 0.07	1.29 ± 0.10
	1		3 h	1.24 ± 0.09	1.20 ± 0.11	1.42 ± 0.016	1.45 ± 0.08
	1		5 h	0.61 ± 0.06	0.79 ± 0.10	0.67 ± 0.06	1.00 ± 0.03
Soman + Multiple Dose HI-6	1[c]	30 – 60 s prior	1/2 h	1.24 ± 0.04	0.96 ± 0.10	1.12 ± 0.058	1.12 ± 0.12
	2	+ 1/2 h	1 1/2 h	1.58 ± 0.15	1.63 ± 0.15	1.59 ± 0.13	1.53 ± 0.10
	3	+ 1 h	3 h	1.57 ± 0.16	1.38 ± 0.15	1.76 ± 0.14*	1.67 ± 0.14
	4	+ 3 h	5 h	0.70 ± 0.05	1.04 ± 0.10	0.77 ± 0.07	1.14 ± 0.11

a AChE expressed as nmol ACh hydrolyzed/mg wet weight/min ± SEM.

b Soman 650 μg/kg, s.c. plus atropine SO_4 17.4 mg/kg, i.p. 30 min prior to soman.

c HI-6 (125 mg/kg) 30 – 60 s prior to soman.

AChE values of all soman groups irrespective of treatment were statistically different from controls.

* Denotes a value statistically different from soman without HI-6 ($p < .05$).

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TABLE 2

THE EFFECT OF SINGLE VS MULTIPLE DOSES OF HI-6 ON RAT DIAPHRAGM ACETYLCHOLINESTERASE

TREATMENT[a]	AChE ACTIVITY[b]	TIME TO SACRIFICE FOLLOWING SOMAN 650 μg/kg			
		1/2	1 1/2	3	5
Control	0.78 ± 0.14 (5)	—	—	—	—
Soman	0.006 ± 0.003 (12)	no survivors	—	—	—
Soman + HI-6 (one dose)[c]		0.10 ± 0.022 (12)*	0.036 ± 0.01 (5)	0.016 ± 0.006 (11)	0.015 ± 0.011 (6)
Soman + HI-6 (multiple doses)[d]		0.10 ± 0.022 (12)*	0.15 ± 0.02 (11)*	0.10 ± 0.019 (11)*	0.031 ± 0.026 (6)

a All animals were pretreated with atropine sulphate 17.4 mg/kg, i.p. 1/2 h prior to soman 650 μg/kg, s.c. ($6 \times LD_{50}$).

b AChE activity expressed as nmol ACh hydrolyzed/mg wet weight/min ± SEM (number of animals).

c 125 mg/kg, i.p. HI-6 given 30 – 60 s prior to soman.

d 125 mg/kg, i.p. HI-6 given as in (c) plus at 1/2 h, 1 1/2 h and 3 h.

* Values significantly different from soman values ($p < 0.05$).

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TABLE 3

AChE ACTIVITY IN FASCICULATING AND NONFASCICULATING MUSCLE GROUPS AND BRAIN FROM SOMAN TREATED RATS GIVEN HI-6

TISSUE TYPE	T.F.L.	% DECREASE	RECTUS ABDOMINIS	% DECREASE	BRAIN	% DECREASE
Control[a]	0.37 ± 0.020	—	0.67 ± 0.017	—	8.44 ± 0.21	—
Fasciculating[b]	0.0021 ± 0.0019	99.4	no detectable activity	100	0.94 ± 0.022	88.8
Nonfasciculating[c]	0.0059 ± 0.0027	98.4	0.0086 ± 0.004	98.7	1.08 ± 0.085	87.1

a Animals given 17.4 mg/kg atropine SO_4, 30 min prior to soman 650 μg/kg, AChE activity expressed as nmol ACh hydrolyzed/mg wet weight/min ± SEM.

b Treated as above with HI-6 30 – 60 s prior to soman and sacrificed 2 h later.

c Treated as b at 2 h an additional dose of HI-6, 125 mg/kg, i.p. was given and animals were sacrificed 5 min later.

Number of tissues in all groups — 6.

Tensor fasciae latae (T.R.L.).

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FIGURE 1

This figure shows the percent survival over time of atropine pretreated rats given one of two doses of soman and either one or multiple doses of HI-6 (125 mg/kg, i.p.). Atropine pretreated rats died after 650 μg/kg soman in about 3 min (■). When one dose of HI-6 was given 30 – 60 s prior to soman (650 μg/kg (○) or 900 μg/kg (●) the animals survived up to 6 h. When additional HI-6 was given at 1/2, 1 1/2, 3 and 6 h post soman 100 percent of the animals survived 14 days at 650 μg/kg (△) while 100 percent survived 900 μg/kg for 6 h but none survived 14 days (□). A third group of animals (n = 12) was given additional HI-6 (▲) at 1/2, 1, 2, 3, 4, 5 and 6 h resulting in a survival rate of about 60 percent after 14 days.

FIGURE 2

2A - C. Tremor parameters were measured in a group of atropine pretreated rats given one dose of HI-6 30 – 60 s before 650 μg/kg soman (○) and compared to the tremor activity measured at 2 h in animals given subsequent injections of HI-6 at 1/2 h and 1 1/2 h (●). All means of the upper line (○) are significantly different from those in the lower line ($p < .05$).

2D - F. Tremor parameters were measured as above 2 h after a single injection of HI-6 (125 mg/kg, i.p.) and 650 μg/kg soman. After 5 min of recordings the rats were given a second dose of HI-6 and the parameters of tremor followed for a further time period. Each point represents the mean ± SEM of 6 animals. All parameters of the tremor activity were markedly lower following multiple HI-6 injections. Each point is the mean ± SEM of the measurements.

FIGURE 3

Rats were given atropine and soman as outlined in previous Figures. After 2 h they were given an injection of diazepam 2.5 mg/kg, i.p. and the tremor activity measured for 10 min. No statistically significant alteration in tremor pattern was measured following diazepam but a second dose of HI-6 (125 mg/kg, i.p.), 10 min after diazepam produced dramatic significant decreases in tremor activity for the time period measured. Each point is the mean ± SEM (n = 6).

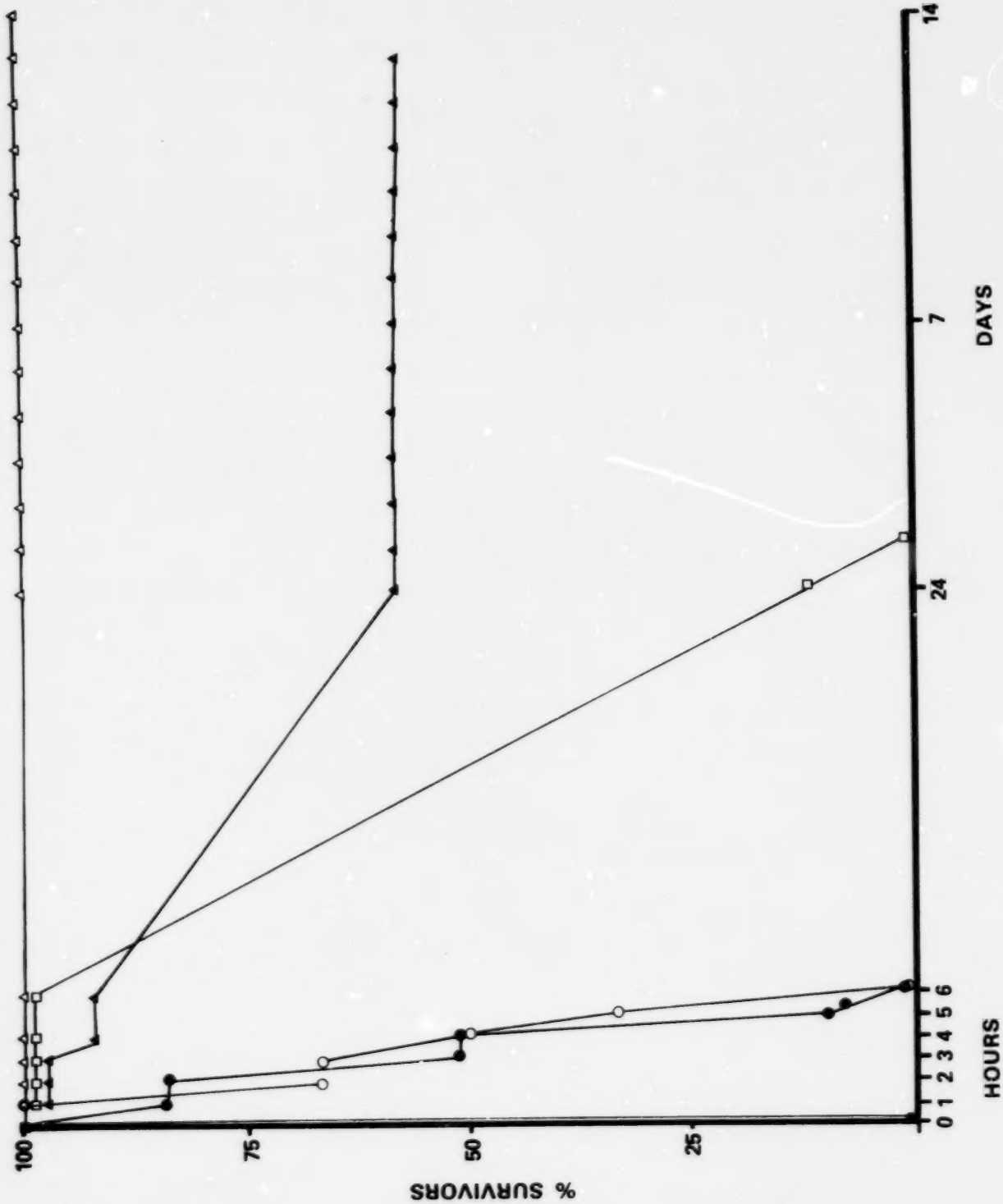

FIGURE 1

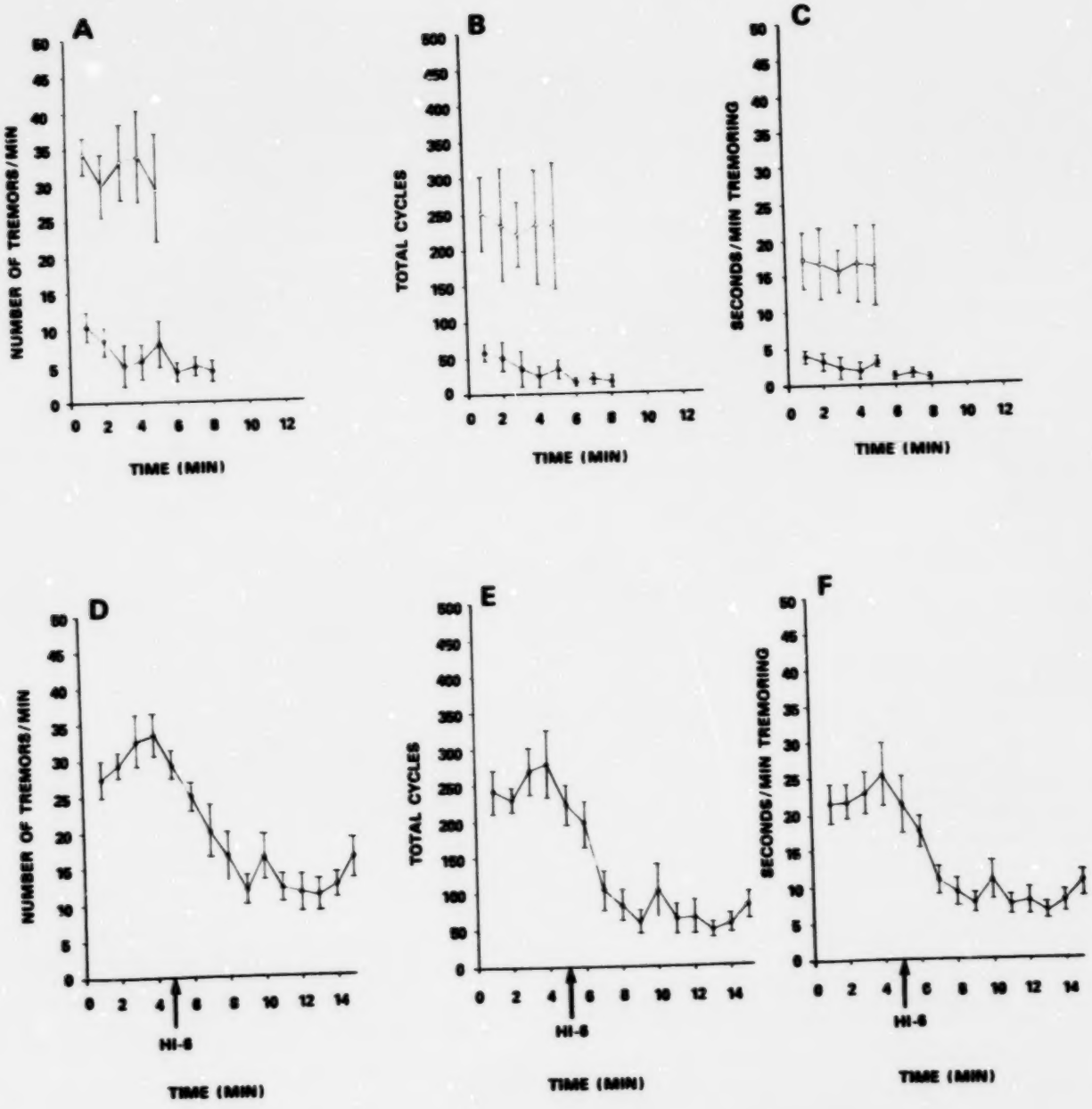
A
NUMBER OF TREMORS/MIN
TIME (MIN)
B
TOTAL CYCLES
TIME (MIN)
C
SECONDS/MIN TREMORING
TIME (MIN)
D
NUMBER OF TREMORS/MIN
HI-6
TIME (MIN)
E
TOTAL CYCLES
HI-6
TIME (MIN)
F
SECONDS/MIN TREMORING
HI-6
TIME (MIN)

FIGURE 2

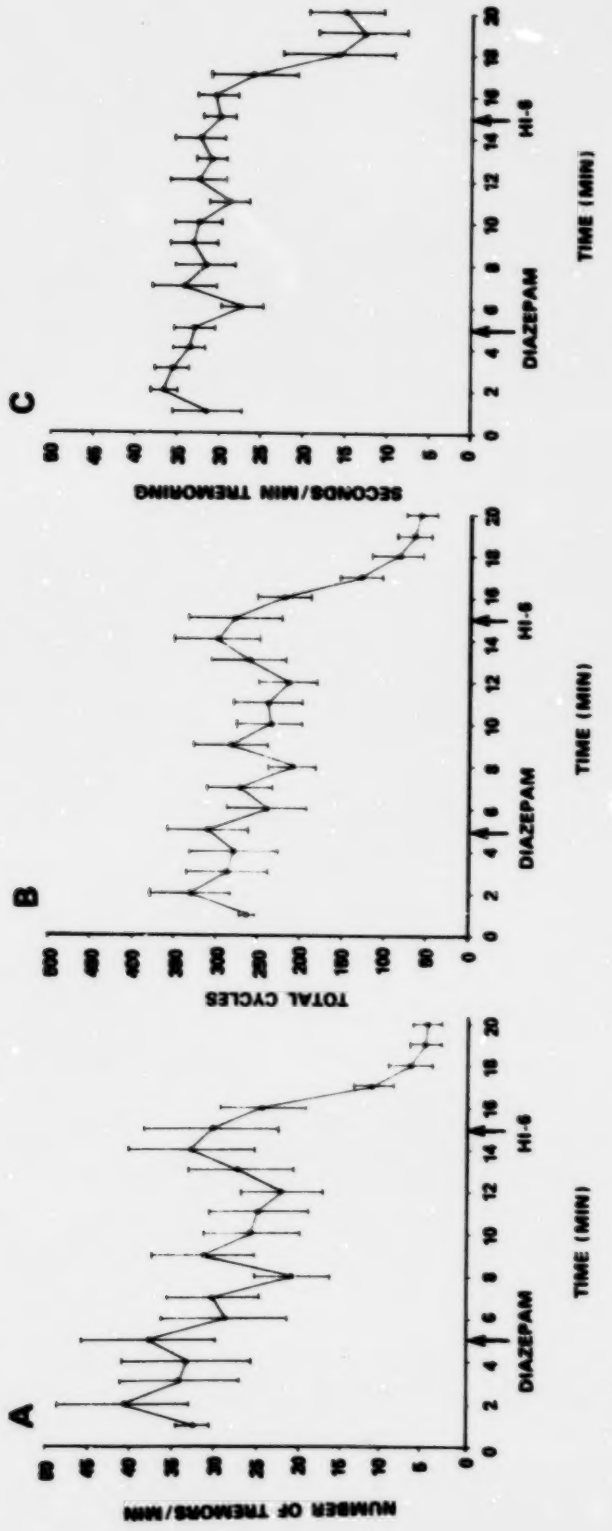
A
NUMBER OF TREMORS/MIN
DIAZEPAM
HI-6
TIME (MIN)
B
TOTAL CYCLES
DIAZEPAM
HI-6
TIME (MIN)
C
SECONDS/MIN TREMORING
DIAZEPAM
HI-6
TIME (MIN)

FIGURE 3

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Atropine pretreated rats given soman (650 μg/kg) survived an average 3.2 min. HI-6 (125 mg/kg i.p.) increased survival time of all animals usually 3-6 hrs while multiple doses of HI-6 given post soman completely protected rats for at least 14 days. Soman treated animals given one dose of HI-6 developed short-lasting convulsions, followed by a convulsion free period then in about 1½ hrs by a gradual return of severe tremors. A second dose of HI-6, unlike diazepam, quickly abolished the tremors. Diaphragm AChE activity was higher than control values following 1 dose of HI-6 ½ hr post soman but not at subsequent time intervals, while diaphragms from animals treated with HI-6 several times were higher than controls for at least 3 hrs. AChE activity remained virtually unchanged in all brain areas examined following either regimen. Skeletal muscles removed from animals which had developed delayed tremors had virtually no AChE activity nor did muscles from animals given a second dose of HI-6 which stopped the tremors. The results indicate that multiple HI-6 doses afford much better protection from soman than one dose. This enhanced protection may be due partially to the maintenance of AChE activity in the diaphragm for long periods and additionally to a direct effect on skeletal muscle resulting in improved respiratory function.

These results indicate that HI-6 may be even more useful against soman poisoning than previously recognized if the dose schedule is altered. When future studies are planned to test the effects or effectiveness of HI-6 in other species or in human trials, the factor of multiple injections over long periods of time should be a major consideration.

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protective effects
acetylcholinesterase
tremors
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